J. Glob. Innov. Agric. Soc. Sci., 2017, 5(2):43-46
ISSN (Online): 2311-3839; ISSN (Print): 2312-5225
http://www.jgiass.com

SULPHURIC ACID, COMMERCIAL BLEACH AND PLANT PRESERVATION MIXTURE –PPM USED IN COMBINATION FOR SURFACE STERILIZATION OF SUGAR BEET SEEDS

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The sugar beet (*Beta vulgaris* L.) grows all over Turkey and has major contribution to the local sugar demands. Sugar beet seeds have hard serrated, irregular structure that makes it very difficult to sterilize and carry out plant tissue culture studies. This study aimed to optimize sterilization procedure for monogerm cv. Diamenta, Polygerm line EMU 8 and monogerm line SG3 of sugar beet (i) using 80 min treatment with sulphuric acid singly or commercial belach singly, (ii) 80 min sulphuric acid treatment followed by sterilization with 100% commercial bleach (containing 5% NaOCl) and (iii) 80 min sulphuric acid treatment followed by use of 100% commercial bleach (containing 5% NaOCl) and 3% PPM (in the same order) both under dark and 16 h light period. The results of the study showed that third method of sterilization was the most effective to sterilize seeds both under dark and 16 hours light photoperiod conditions. Germination percentage of the seeds varied under two conditions. Higher germination was noted in the dark compared to light. The seed germination percentage was in line with the tetrazolium seed viability test. It was concluded that the method could be used safely for surface sterilization of sugar beet seeds.

Keywords: PPM, sulphuric acid, *in vitro*, surface sterilization, commercial blelach, sugar beet.

INTRODUCTION

The sugar beet (*Beta vulgaris* L.) is major crop that contributes largely to national sugar consumption in Turkey and is grown in 59 provinces with a production of 19465452 tons beet and grown over an area of 3224477 decares during 2016 (TUİK 2016). The annual sugarbeet production fluctuates depeding on various biotic factors like insect pests, fungal bacterial and viral infestations and abiotic factors heat, drought, salt tolerance etc (Kahriz 2017).

There is need to introduce biotechnological approaches to reduce breeding time of sugar beet and develop sugar beet cultivars resistant to these inhibiting factors; as traditional methods of propagation are very time and labor consuming. Plant cell and tissue culture offers an alternative to accelerate breeding compared to traditional techniques of plant breeding in biennial sugar beet crop. It make use of steril explants obtained from stem nodes, petioles, leaves, roots or callus grown under aseptic conditions with known environment and nutritient elements (Kahriz 2016, Zilkah *et al.,* 1997a,b,c).

Sugar beet seeds are irregular and have hard-shelled, pericarp and embryo in fruit/seed cavity with serrated shape. This makes both germination and surface sterilization of the seeds very difficult. Surface sterilization is the most important step in the tissue culture of plants. Therefore, the aim of this study was to optimize a standardized surface sterilisation procedure enabling ample provision of sterilized seedlings for use in sugar beet tissue culture, breeding and transformation programs.

***Experimental material*:** The experiment made use of sugar beet monogerm cv. Diamenta, Polygerm line EMU 8, and monogerm line SG3 that were were obtained from the Sugar Institute in Etimesgut, Ankara, Turkey.

The cv. Diamenta is resistant to Rhizomania. It could be machine harvested and was developed by the Sugar Research Institute, Etimestgut during 2011. It has high root yield of 9,1 tons/decare with >18% sugar. Sugar beet Polygerm line EMU 8 and line SG3 are resistant to Rhizomania disease with high root yield and >18% sugar percentage (Kahriz 2016).

Sugar beet seeds belonging to any genotype are irregular, have hard-shelled, pericarp and embryo in fruit/seed cavity with serrated shape. Because of the hard serrated structure of the seeds both germination and surface sterilization are very difficult.

After conducting number of optimization methods (not reported) three methods were selected to determine the best surface sterilization procedure for using the seeds belonging to the three genotypes for use in tissue culture studies in separate experiments to be carried out later on.

Treatment with sulphuric acid: The seeds of the three genotypes were treated with sulphuric acid for 80 min. and rinsed with 3 × 3 min with bidistilled sterilized water.

Treatment with commercial bleach: The seeds of the three genotypes were treated with 100% commercial bleach for 10 min. and rinsed with 3 × 3 min with bidistilled sterilized water.

Treatment with sulphuric acid and commercial bleach: The seeds of the three genotypes were treated with sulphuric acid

for 80 min as mentioned earlier followed by rinsing with 3 × 3 min with bidistilled sterilized water. Subsequently, these seeds belonging to each genotype were treated with 100% concentration of commercial bleach (5%NaOCl –Ace Turkey) for 10 and and re rinsed 3 × 3 min with bidistilled sterilized water.

Treatment with PPM: The seeds of the three genotypes were treated with 3% (v/v) PPM in water for 12 h. by soaking.

Treatment with sulphuric acid, commercial bleach and PPM (Plant Preservation Mixture): First of all the seeds of each genotype were treated with sulphuric acid for 80 min and rinsed 3 ×3 min with with bidistilled sterilized water to remove traces of the acid. Thereafter, the seeds of each genotype were treated with 100% concentration of commercial bleach (5%NaOCl –Ace Turkey) for 10 min and rinsed 3 × 3 min with with bidistilled sterilized water to remove traces of the bleach. Thereafter, the seeds were transferred to 3% (v/v) PPM (Plant Preservation Mixture) in water, for 12 hours by soaking.

Double distilled water was used in the preparation of the MS medium containing 30 g/l sucrose and solidified with 6.5 g/l agar. After the pH of the nutrient medium was adjusted to 5.8 ± 0.1 using 1N NaOH or 1N HCl. Before adding agar, it was autoclaved to 120 °C for 20 min at 1.45 kPa atmospheric pressure.

The surface sterilized seeds belonging to each of the above mentioned experiments were incubated under 16 h lights (35µ mol photons/m^2/s) photoperiod using white fluorescent lights at 24 °C in sterile Petri dishes to determine the effectivity of the respective treatments against disinfection followed by their ability to germinate the seeds after six days of initial culture both under dark and light conditions.

***Seed Viability Test*:** 2,3,5 triphenyltetrazolium chloride (TTC) method proposed by Anonymous (1999) was used for determination of seed viability of each genotype. In this study, each of the sterilized sugar beet seeds of the three genotype (as per third method of disinfection) were cut in to two using a sharp knife scalpel blade and treated with the 1mg/ml (w/v) tetrazolium salts. The seeds were incubated in dark at room temperature (24 ± 1 ° C) for 24 hours to develop red formazan colour. TTC is reduced to red formazan under dark after reaction with living celss, which is directly proportional to the viable active cells. Therefore, the tetrazolium test is considered a comparatively fast method for evaluating the viable embryos. Tetrazolium test needs less than 12 h to attain susceptibility and Show formation of formazan.

***Statistical analysis*:** All experimental treatments were evaluated twice using completely randomized design. All data was analysed using one way ANOVA and the the significant differences among means were compared by Duncan Multiple Range test of SPSS 22. All percentage data was arc sin transformed before statistical analysis (Steel and Torrie 1980).

RESULTS AND DISCUSSION

***Tetrazolium test*:** Tetrazolium test showed seed viability of 50% each for cv. Diamenta and EMU 8. Wheras, seed viability of 64. % was confirmed for line SG3.

***Surface disinfection and seed germination*:** Contamination of seeds and culture media pose major threat in plant cell and tissue culture studies. A number of methods have been tried to disinfect plant tissue culture material including use of fungicides (Heldmen *et al.*, 1987) and antibiotics (Kneifel and Leonhardt 1992, Leifert *et al.,* 1992) and disinfectants like NaOCl (Kahriz and Kahriz 2017), sulphuric acid (Garner and Sanders 1932 published on line 2009). In line with the previous studies, three experiments were conducted after number of optimization experiments for the surface sterilization of the seeds of the monogerm cv. Diamenta polygerm line EMU 8, and monogerm line SG3 of sugar beet.

***Treatment with sulphuric acid, commercial bleach, PPM singly*:** It was not possible to disinfect the seeds using sulphuric acid or commercial bleach simgly. Huge infestation of *Fusarium oxysporium* contamination was noted on all seeds of three genotypes after six days with germination of variable number of seeds. Sugar beet seeds are ideal place for hibernating fungus and bacterial spores as they are round in shape and have a mosaic / rough indented protruding structure., it was not effective seed disinfection method due to irregular and serrated shape of seeds that hibernated fusarium spores that could not be reached by the acid. It was concluded that although, 80 minutes scarification with sulphuric acid was effective to soften the hard seed coats and make them water permeable enabling the them to germinate; it was not effective to disinfect them. Once the seeds were cultured on MS medium, containing 30 g/l sucrose, these spores multiplied rapidly and infested the germinating seedlings or seeds leading to either death or stunted growth. All of the stunted seedlings showed damping off with in 3-5 days after emerging from the seeds shells. No differences were observed among three genotypes in terms of disinfection both under the dark or 16 hours light photoperiod. The results of acid scarification are partially in agreement with Garner and Sanders (1932 published on line in 2009); who report that treatment of sugar-beet seeds with sulphuric acid leads to an increase in rate of germination. They attribute the increase in germination to a greater permeability of the hard seed coats, which aids faster germination.

Commercial bleach failed to disinfect, scarify and germinate the seeds. It induced huge infestations of fussarium colonies. Commercial bleach scarification is effective only if given prolonged treatment. This study made use of 10 min sterilization/scarification treatment for very hard seeds. Galletta *et al.,* (1989) recommend scarification with 5% commercial bleach for 48 or 96 h for Rubus; whereas, Campbell *et al.,* (1988) recommends scarification with 15% NaOCl for 18 h for blackberry.

PPM used singly disinfected the seeds of three genotypes but failed to germinate them for want of hard seed coat scarification, as the PPM is a biocide, disinfectant and could not be used to scarify the seeds. Active ingredients of PPM are methylisothiazolinone, magnesium chloride, magnesium nitrate, sodium benzoate and potasium sorbate (Guri and Patel 1998) that are not suitable to scarify the seeds.

***Treatment with sulphuric acid and commercial bleach*:** Surface sterilization was not achieved on three genotypes with huge infestations of fusarium wilt without any bacterial contamination surrounding the seeds in 4-5 days of culture. Due to huge fungal infestation; this method of disinfection was also not considered suitable for sterilization of sugar beet seeds. No difference was observed between the seeds treated under light and dark conditions. The reults of the experiment are in partial agreement with Petrus–Vancea et al., 2009; who found that the sugar beet seed treatment with sodium hypochlorite, was insufficient to sterilize them with poor germination percentage in range of 25-32.5%, on four sugar beet cultivars with development of infections as the culture medium rich in sucrose was prone to multiply even very small amounts of dormant fungal spores very rapidly.

Treatment with sulphuric acid, commercial bleach and PPM (Plant Preservation Mixture): It was possible to achieve surface sterilization with this treatment. The results of the study showed significant differences in the rate of germination among the seeds of three genotypes both under dark and light conditions. Duncan multiple range test results showed seed germination percentage of 40.00%, 68.33% and 33.33% and 45%, 50.00%, and 61.67% for mono germ cv. Diamenta, line EMU 8 and line SG3 of sugar beet under 16 hours light and dark conditions respectively (Table 1). Seed germination under dark conditions confirms results of tetrazolium test.

Table 1. Germination rates related to dark and 16 hour light photoperiod on the cv. Diamenta, lines EMU 8 and SG3 of the sugar beet and after six days of culture

Sugar beet cultivars /lines	Seed germination (%) after incubation under 16 h light photoperiod **	Seed germination (%) after incubation under dark conditions**
Diamenta	40.00b	45.00c
EMU 8	33.33c	50.00b
SG3	68.33a	61.67a

**There was a significant difference among means at 0.01 level of significance indicated by different letters in the same column using LSD test

No fungal and bacterial contamination were noted on any culture. The germinated seedlings achieved length of 2-3 cm on cv. Diamentha, 4-5 cm on line SG3 and 2-3 cm on line EMU 8 after 7-9 days of culture. PPM has been reproted to be a powerful biocide and disinfectant of plant materials by Niedz (1998) and Compton and Koch (2001).

The results of the study are in agreement with Guri and Patel (1998); who claim that PPM is a heat stable broad spectrum biocide that could act as powerful disinfectant of plant tissue culture material. PPM target microorgansims with minimum effect on seed germination and is not toxic if used in concentrations of 0.5-1 ml/L. Some researchers even recommend seed sterilization with PPM (Jain *et al.,* 2005, Nail and Roberts 2005, Jiménez *et al.,* (2006). Paul *et al.,* (2001). and. Rowntree (2006) singly and combined with other sterilization methods.

Information on safe use of PPM for callus growth and adventitous shoot regenration in range of 0.5-5 ml/L is available (Niedz 1998, Compton and Koch 1998). Use of PPM above this concentration could act as toxic on the growth of callus and regenerated plants.

***Conclusion*:** No information is available on use of sulphuric acid, NaOCl and PPM in conjunction, this study demonstrate that PPM can be used safely for disinfection and germination of sugar beet seeds under *in vitro* conditions.

Acknowledgement: The authors acknowledge the guidance by Prof. Dr. Khalid Mahmood Khawar of Ankara University, Turkey in carrying out the research and preparation of the manuscript.

REFERENCES

Campbell, P.L., D.J. Erasmus and J. van Staden. 1988. Enhancing seed germination of sand blackberry. Hort. Science. 23: 560–561.

Compton, M.E. and J. M. Koch. 2001. Influence of plant preservative mixture (PPM)TM on adventitious organogenesis in melon, petunia, and tobacco. *İn vitro* Cell. Dev. B.37:259-261. ·

Galletta, G.J., J.R. Ballington and A.D. Draper. 1989. Pregermination treatment of seeds of species and hybrids in *Rubus* with sodium hypochlorite. Acta Hort. 262: 289–296.

Garner, F.H. and H. G. Sanders.1932 published on line in 2009.Investigations in crop husbandry. I. The effects of seed treatments on the germination and yield of sugar beet. J. .Agri. Sci. 22 : 551-559.

Guri, A.Z. and Patel, K.N. 1998. Compositions and methods to prevent microbial contamination of plant tissue culture media. US patent. 5:750,402.

Heldman, J. and D. Lancet. 1986. Cyclic AMP-dependent protein phosphorylation in olfactory cilia. J. Neurochem. 47: 1527-1533.

Jain, S.C., B. Singh and R. Jain. 2000. Arnebins and antimicrobial activities of Arnebia hispidissima DC. cell cultures. Phytomedicine. 6: 474-476.

Jiménez, V.M., J. Castillo, E. Tavares, E. Guevara and M. Montiel. 2006. *İn vitro* propagation of the neotropical giant bamboo, Guadua angustifolia Kunth, through axillary shoot proliferation. Plant Cell Tiss. Org. Cult. 86: 389-395.

Kahriz, P.P. and M. P. Kahriz. 2016. Somatic Embryogenesis on Plumule and Radicle Explants Obtained from Warm Water Hydroprimed Wheat (*Triticum aestivum* L.) cv. Kunduru and cv. Cakmak Seeds. Sains Malaysiana. 46: 35–41.

Kahriz, P.P. 2016. *Agrobacterium* mediated genetic transformation of sugar beet (*Beta vulgaris* L.) against herbicide resistance genes. PhD thesis. Department of Field Crops. Institute of Natural and Applied Sciences. Ankara. Üniversity. Turkey.

Kneifel, W. and W. Leonhardt. 1992. Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture. Plant Cell Tiss. .Org. Cult. 29: 139-144.

Nail M.C. and S.C. Roberts. 2005. Culture of isolated single cells from Taxus suspensions for the propagation of superior cell populations. Biotec. Lett. 27: 1725-1730.

Niedz, R.P. 1998. Using izothiazolone biocides to control microbial and fungal contaminants in plant tissue culture. Hort. Technolgy. 8: 598–601.

Paul, A., C. Semer, T. Kucharek, and R. J. Ferl 2001. The fungicidal and phytotoxic properties of benomyl and PPM in supplemented agar media supporting transgenic arabidopsis plants for a Space Shuttle fl ight experiment. Appl. Microbiol. Biotechnol. 55: 480-485.

Petrus–Vancea, A., N. Palcuţ and A. Baciu. 2009. Sugar beet (*Beta vulgarıs* L. var. Saccharifera) vıitroculture initiation from encapsulated seeds. Analele Universităţii din Oradea, Fascicula Biologie. XVI: 91-93.

Rowntree, J.K. 2006. Development of novel methods for the initiation of *in vitro* bryophyte cultures for conservation. Plant Cell Tiss. Org. Cult. 87: 191-201.

Steel, R.G.D. and J.H. Torrie. 1980. Principles and procedures of statistics: A biometrical approach. 2nd ed. McGraw Hill Book Co. New York.

Tuik 2016. http://www.tuik.gov.tr (accessed 1.12.2016)

Zilkah, S., J. and Gressel, 1977b. Cell cultures vs. whole plants for measuring phytotoxicity. I. The establishment and growth of callus and suspension cultures and definition of factors affecting toxicity. Plant Cell Physiol. 18: 641–655

Zilkah, S., J. and Gressel. 1977c. Cell cultures vs. whole plants for measuring phytotoxicity. III. Correlations between phytotoxicities in cell suspension cultures, calli and seedlings. Plant Cell Physiol. 18: 815–820.

Zilkah, S., P.F. Bocion, and J. Gressel. 1977. Cell cultures vs. whole plants for measuring phytotoxicity. II. Correlation between phytotoxicity in seedlings and calli. Plant Cell Physiol. 18: 657–670.